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Digestive Enzymes and Hormones in Gilthead Seabream Larvae (*Sparus aurata*) Fed *Artemia* Nauplii Enriched with Free Histidine

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Abstract

The digestive enzymes and hormones of gilthead seabream (*Sparus aurata*) larvae fed live prey (*Artemia* nauplii) enriched with free histidine were investigated for 16 days (from day 24 to day 40 after hatching). Larvae were sampled at 4-day intervals. The control group had significantly lower growth than the enriched group ($p < 0.05$). Trypsin activity was higher in the control ($p < 0.05$). Bombesin activity significantly differed between treatments, periods, and period x treatment interactions and the cholecystokinin level was significantly higher in the enriched groups ($p < 0.05$). Results indicate that *Artemia* nauplii can successfully be enriched with free histidine, bombesin and cholecystokinin activity can be stimulated by free histidine, and mechanisms controlling the adaptation of trypsin activity to the amount of dietary protein were not activated within the 40-day study period.

Introduction

Marine fish larvae are immature at hatching. During their first few weeks, they undergo major developmental changes. Their digestive tract is still developing and the stomach is absent until they approach or attain metamorphosis. During this time, regulation of proteolytic enzyme secretion during feeding is controlled by several components. Digestive hormones are key factors in the gastrointestinal function of vertebrates (Bentley, 1998). Nutrients such as free amino acids (FAA) and free fatty acids (FFA) entering the mammalian digestive tract stimulate endocrine responses

that control digestion and nutrient assimilation and influence feeding behavior and food intake. Not only do free amino acids fuel the anabolic and catabolic pathways of growing larvae, but they also may trigger the digestive endocrine axis (Chan and Hale, 1992).

The pituitary neuropeptides, bombesin (gastrin-releasing peptide; GRP) and cholecystokinin (CCK), are integral parts of this gastro-entero-pancreatic endocrine system (Moons et al., 1992; Himick and Peter, 1994ab). Bombesin influences digestion by activating the peristaltic movement of the gut

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and the release of HCl as well as increasing blood circulation to the gut wall (McDonald et al., 1979). Cholecystokinin stimulates gall-bladder contraction and secretion of pancreatic enzymes including trypsinogen, the precursor of the key proteolytic enzyme, trypsin (Vander et al., 1998). Trypsin serves as a key enzyme since it activates other proteolytic enzymes upon entrance into the gut lumen (Holst and Schmidt, 1994; Liddle, 1995).

In marine fish larvae, the delivery of amino acids from intestinal protein digestion may be insufficient to supply their FAA needs. Rotifers and *Artemia*, commonly used in aquaculture as feeds for fish larvae, are apparently deficient in one or more of the following amino acids, depending on the stage of larvae development: histidine, sulfur amino acid, arginine, lysine, or valine (Aragao et al., 2004). *Artemia* nauplii contain markedly lower levels of free amino acids than wild copepods (Fyhn et al., 1993). One way of compensating for deficient amino acids in live feeds such as rotifers and *Artemia* is supplementation with the limiting amino acid.

Histidine is the limiting amino acid for white seabream (*Diplodus sargus*) larvae (Saavedra et al., 2006). Thus, the aim of this study was to determine the effect of feeding *Artemia* enriched with free histidine on levels of digestive enzymes and hormones in gilt-head seabream larvae.

Materials and Methods

Experimental animals and culture conditions. This study was carried out at the Mediterranean Fisheries Research, Production and Training Institute. Fertilized eggs were collected from broodstock tanks and incubated in conical fiberglass tanks at 16°C. For the experiment, newly-hatched larvae (approximately 100 larvae/l) were transferred to 300-l fiberglass tanks with black walls. Running sea water, filtered through a UV filter, was exchanged by green water (*Nannochloropsis* sp.) at a rate of 10% every day. Air and fresh sea water were introduced into the bottoms of the tanks to prevent water stratification. For the first four days after hatching, the temperature was kept at 16°C±0.5°C, for the following

16 days at 18°C±0.5°C, and for the final 20 days at 20°C±0.5°C. Water temperature was controlled by pipe heating systems and automatic transformer equipment was calibrated at ±0.5°C. Salinity was 35-38 g/l throughout the experiment. Oxygen levels were maintained above 6.5 mg/l with liquid oxygen systems. Light (100-150 lux maximum at the water surface) was applied 24 h a day.

Feed regime. The feed regimes of the experimental and control groups were identical for the first 24 days and consisted of *Brachionus plicatilis* on days 4-20 (maximum concentration 10 prey/ml) and *Artemia* nauplii from day 15 onwards (maximum density 2 prey/ml). On days 25-40, the experimental groups were fed *Artemia* metanauplii enriched with free histidine (maximum 2 prey/ml) while control larvae were fed unenriched *Artemia* metanauplii. The rotifers were reared on baker's yeast and enriched with Selco (INVE Products) prior to transfer to the experimental tanks. *Artemia* cysts were incubated in continuously aerated and illuminated filtered sea water at 30°C, collected after 24 h, and washed with tap water. Nauplii were enriched by introducing 600 nauplii/ml into a 15-l round bottom beaker containing 12 l filtered sea water and a 5.3mM FAA solution for 16 h. Gentle aeration ensured homogeneous distribution of the nauplii. Each treatment was conducted in triplicate.

Sampling. Larvae were sampled four times at 4-day intervals to monitor growth. Three samples (40 larvae/sample) were taken from each group and larvae were weighed on a microbalance (Precisa XB 220A) to an accuracy of ±0.1 mg. In addition, to determine enzymatic and cholecystokinin (CCK) activities, three samples (50 larvae/sample) were taken from each group before morning food distribution and immediately stored in liquid nitrogen (-196°C) to prevent protein autolysis. Larvae were sampled for bombesin activity 5 min and 15 min after the start of feeding and immediately stored in liquid nitrogen (-196°C) to prevent protein autolysis.

Enzyme analyses. Whole body larvae were homogenized in 5 volumes (v/w) of ice cold distilled water. Soluble protein was deter-

mined according to Bradford (1976). Amylase and trypsin activities were assayed according to Metais and Bieth (1968) and Tseng et al. (1982), respectively. Alkaline phosphatase (AP), leucine aminopeptidase N (lap), and leucine alanine peptidase (leu-ala) were assayed according to Bessey et al. (1946), Maroux et al. (1973), and Nicholson and Kim (1975), respectively. Amylase activity was expressed as the equivalent enzyme activity required to hydrolyze one mg of starch in 30 min at 37°C. Enzyme activity was expressed as μ moles of substrate hydrolyzed per minute per mg protein (i.e., U/mg protein) at 37°C for AP and lap and at 25°C for trypsin while leu-ala peptidase activity was expressed as nmoles of substrate hydrolyzed per minute per mg protein (i.e., U/mg protein) at 37°C. The amino acid composition of *Artemia* enriched with free histidine was analyzed according to AOAC (1995).

Hormone analyses. Lyophilized larvae were transferred to tared Eppendorf tubes and their weights determined using a microbalance (Precisa XB 220A) to an accuracy of ± 0.1 mg. The tubes were then submerged in liquid nitrogen and the frozen tissues were pulverized with a fitted motorized pestle. One milliliter of methanol was added to the pulverized samples which were vortex-mixed for 30 s and left at least 30 min on the bench at 4°C for extraction. The tubes were centrifuged at a minimum of 6000 g for 15 min at 4°C and the methanol supernatants were carefully transferred to clean Eppendorf tubes and evaporated using a speed Vac. The remaining dried extracts were used for radioimmunoassay (RIA) CCK. The kit reagents were brought to room temperature before use and reconstituted as specified by the supplier. Standards containing 0.78-25 pmol CCK-8/l were prepared in a diluent buffer (0.05 mol/l, pH 7.4). The dried CCK extracts from the samples of the pooled of gilt-head seabream larvae were solubilized in 0.5-1.0 ml diluent before the assay. Aliquots of 200 μ l of samples and standards were pipetted into triplicate 4.5-ml polystyrene cryotubes. The CCK in the extracts was assayed by competitive RIA using anti-CCK-8 sulfate

and the trace 125 I-CCK-8 (Rojas-Garcia et al., 2001). The antibody-bound tracer was separated from the unbound fraction using double antibody solid phase, and its radioactivity was measured in a gamma counter for 3 min. The CCK content was calculated as pmol/l by interpolation.

Bombesin analysis. The bombesin contents were determined according to Kolkovski (1995). Frozen larvae (previously washed with deionized distilled water and frozen in liquid nitrogen) were defrosted and kept at 4°C. The larvae were mixed with buffer phosphate that included EDTA (7.2 mg/ml) and Trasylol (Sigma, 500 KIU/ml) at the ratio of 1:50 and homogenate using Ultra-turax homogenizer (IKA Ultra Turax T25) with 15 s intervals to prevent a rise in temperature. The homogenate was filtered through sintered glass filter (Kimax #2) and centrifuged (Sorvall Combi Plus, cooler centrifuge) at 25,000 g for 30 min. The supernatant was then frozen at -80°C. Bombesin activity was detected using RIA test kit (Bombesin RIA-7113, Peninsula Laboratories). The antigen in the kit is specific to frog skin originated bombesin. The kit is specific for mammalian gastrin-releasing peptide (GRP) with an efficiency of 50%.

Statistical analysis. Data were analyzed according to a two factors repeated measured treatment plan and differences were considered significant when $p < 0.05$. The Bonferroni test was used to determine differences between period averages. The SPSS 9.0 statistical program was used to evaluate data (SPSS, 1993).

Results

After 16 h of enrichment, the free histidine content in enriched *Artemia* was almost three times as high as in the unenriched control. Larvae fed the enriched *Artemia* had significantly higher weight, protein contents, and leu-ala peptidase but lower amylase, trypsin, and lap values than the control (Table 1). The ratio of activity of the brush border peptidase (lap) to cytosolic peptidase (leu-ala) was significantly higher in the control while bombesin activity and CCK levels tended to be higher in the larvae fed the enriched *Artemia*.

Table 1. Comparisons between larvae of sea bream (*Sparus aurata*) fed *Artemia* nauplii enriched with free histidine and control larvae fed unenriched *Artemia* (means±standard error).

	Sampling day				
	28	32	36	40	Avg
<i>Avg wt (mg)</i>					
Control	2.98±0.006	4.30±0.03	11.53±0.18	26.60±0.01	11.35±2.82 ^a
Enriched	2.95±0.01	4.35±0.04	12.6±0.06	28.58±0.31	12.12±3.07 ^b
<i>Avg length (mm)</i>					
Control	6.92±0.01	7.76±0.08	10.70±0.005	12.91±0.01	9.57±0.71 ^b
Enriched	7.00±0.007	7.68±0.07	10.41±0.01	12.71±0.02	9.45±0.68 ^a
<i>Protein content (µg protein/larva)</i>					
Control	7.85±0.05	8.00±0.002	20.94±0.01	50.50±0.007	21.82±5.24 ^a
Enriched	7.80±0.03	7.99±0.003	21.41±0.005	54.04±0.001	22.81±5.68 ^b
<i>Amylase enzyme (U/mg protein)</i>					
Control	2.68±0.14	22.20±0.28	25.84±0.6	11.00±0.41	15.43±2.77 ^b
Enriched	2.54±0.03	7.97±0.18	12.74±0.20	2.74±0.06	6.50±1.27 ^a
<i>Trypsin enzyme (mU/mg protein)</i>					
Control	108.27±0.55	143.99±0.84	159.60±1.01	138.61±1.23	137.62±5.62 ^b
Enriched	109.20±0.26	115.28±0.16	122.44±0.49	58.85±0.70	101.44±7.55 ^a
<i>Alkaline phosphatase enzyme (AP; mU/mg protein)</i>					
Control	431.60±1.46	513.05±2.09	594.20±64.09	879.10±104.76	604.49±57.19 ^a
Enriched	432.17±0.97	552.04±3.70	718.57±87.57	495.30±58.70	549.52±39.18 ^a
<i>Leucine aminopeptidase N enzyme (lap; mU/mg protein)</i>					
Control	449.17±1.60	782.98±7.50	1046.06±3.15	1378.49±4.37	914.18±102.97 ^b
Enriched	446.67±0.69	820.92±4.90	1072.42±3.83	592.00±1.63	733.00±71.49 ^a
<i>Leucine alanine peptidase enzyme (leu-ala; mU/mg protein)</i>					
Control	351.20±0.65	757.78±2.84	1044.94±3.18	2945.50±3.57	1274.86±300.16 ^a
Enriched	352.48±0.12	1850.22±8.98	3011.92±2.11	1972.75±4.55	1796.84±285.90 ^b
<i>Leucine aminopeptidase N/leucine alanine peptidase (lap/leu-ala x 1000)</i>					
Control	1278.94±4.45	1033.25±9.38	1001.06±0.66	467.99±1.23	945.31±89.21 ^b
Enriched	1267.22±2.39	443.68±1.61	356.05±1.02	300.09±1.30	591.76±118.59 ^a
<i>Bombesin activity 5 min after start of feeding (pg/mg protein)</i>					
Control	0.63±0.01	0.37±0.007	0.30±0.01	0.33±0.006	0.41±0.03 ^a
Enriched	0.37±0.008	0.48±0.003	0.52±0.008	0.41±0.006	0.44±0.01 ^b
<i>Bombesin activity 15 min after start feeding (pg/mg protein)</i>					
Control	0.42±0.004	0.34±0.004	0.21±0.004	0.21±0.009	0.30±0.02 ^a
Enriched	0.52±0.009	0.56±0.007	0.59±0.002	0.54±0.004	0.55±0.01 ^b
<i>Cholecystokinin hormone (fmol/mg dry wt)</i>					
Control	1.55±0.02	1.56±0.01	1.70±0.15	1.51±0.13	1.58±0.05 ^a
Enriched	1.53±0.006	2.22±0.004	2.41±0.09	1.82±0.03	2.00±0.10 ^b

Different superscripts in the last column indicate a statistical difference between averages for the treated larvae and the control.

Discussion

The duration of the experiment was long enough to show the effects of free histidine on the digestive enzymes and hormone activity of seabream larvae. On day 40, after 16 days of consuming histidine-enriched *Artemia*, the average weight of the enriched groups was higher than that of the control, indicating that enriching *Artemia* nauplii with free histidine has a positive effect on seabream growth.

In general, amylase activity is stimulated by dietary change. The dietary starch content can modulate changes in amylase activity (Peres et al., 1998). The starch contents of the enriched and the control *Artemia* were similar. Therefore, the difference between groups in amylase activity was not due to dietary components but to genetic programming during larvae development.

Trypsin levels are related to protein content in the lumen (Tseng et al., 1982) whereas this regulatory process may not be functional in younger larvae (Peres et al., 1996). Our results revealed that tryptic activity was higher in the control group than in the enriched group throughout the study, indicating that the mechanisms controlling the adaptation of trypsin activity to the amount of free histidine were inactive within 40 days from hatching.

Alkaline phosphatase (AP) is stimulated by phosphorylated substrates such as phosphoproteins and phospholipids (Shirazi et al., 1978). There was no difference in AP between the enriched and the control groups since both enriched and unenriched *Artemia* contain approximately 1% organic phosphorus.

To follow the development of intestinal digestive function, we studied two peptidases found mainly in two areas: leu-ala, 90% of which is found in the cytosol, and lap, found in brush border membranes (Nicholson et al., 1974). The drop in leu-ala in the enriched groups from day 36 to day 40 suggests a decrease in intracellular peptide digestion accompanying the maturation of enterocytes, a phenomenon described in mammals during the first post natal week (Henning, 1987). In contrast, the continual rise of leu-ala in the control shows that their mode of digestive function remained the same up to day 40.

However, since physiological parameters vary during ontogenesis, the activity of leu-ala alone is not enough to indicate the digestive maturity of the enterocyte. The comparison of peptide digestion in the brush border membranes and peptide digestion in the intracellular compartment (lap/leu-ala) is a better indicator of digestive capacity (Cahu and Zambonino Infante, 1995). The histidine supplementation had a positive effect on leu-ala peptidase activity but not on lap activity. Although there was a sharp drop in leu-ala on day 40 in the enriched group, this enzyme failed to show the expected decrease throughout the experiment, similar to the findings of Kvale et al. (2007). The higher lap/leu-ala ratios in the control are consistent with the rise in the digestive capacity of the brush border membranes (lap) during larvae development. On day 40, the ratio in the enriched groups was lower than in the control, indicating that development of the digestive capacity in the enriched group was slower than in the control. In other words, the enriched *Artemia* did not adequately stimulate intestinal maturation although lap/leu-ala consistently dropped in the control, especially from day 36 to day 40.

Studies on humans show that the most potent stimulants of CCK secretion are the partial digestion products of fat and protein, including di- and tri-peptides (Liddle, 2000). However, research is inadequate when fish larvae are concerned. A study on the first-feeding stages of Atlantic herring reported that soluble protein caused a more rapid and greater increase in CCK content (whole body homogenate) than free amino acids (FAA) and that tryptic activity was higher in larvae fed protein, while no changes occurred in larvae fed FAA (Koven et al., 2002). In contrast, a study on sea bass (*Dicentrarchus labrax*) found that trypsin secretion was stimulated in larvae fed a mixture of FAA while a protein hydrolysate (casein) actually reduced secretion (Cahu and Zambonino Infante, 1995). Digestive end-products such as L-lysine can act directly on pancreatic acinar cells to stimulate enzyme secretion (Grendell and Rothman, 1981) and FAA can stimulate CCK secretion (Liddle, 2000). Atlantic herring larvae, tube-fed a physiological saline, did not

display a CCK response despite the presence of peristaltic movements and apparent fullness of the gut (Koven et al., 2002), indicating that distension of the gut wall is not a trigger for CCK synthesis. Likewise, our results on day 40 reveal that CCK was triggered by the histidine enrichment of the *Artemia*.

The bombesin level in seabream larvae fed *Artemia* nauplii as their sole food was 300% greater than the bombesin level in larvae fed a microdiet but the nutrient factors in *Artemia* responsible for eliciting this endocrine response were not determined (Kolkovski et al., 1997). In our study, there were significant differences in the bombesin level between the enriched group and the control. However, the free histidine content in the *Artemia* nauplii cannot explain this endocrine response.

Three main findings are presented in this study. First, *Artemia* nauplii can successfully be enriched with free histidine. Second, bombesin (GRP) and CCK activity can be stimulated by free histidine. Third, mechanisms controlling the adaptation of trypsin activity to the amount of dietary protein are not activated within 40 days of hatching. Based on these findings, we hypothesize that if the mechanism controlling trypsin activity can be initiated earlier, then live prey can be replaced by microdiets. However, our understanding of the digestive enzymes and hormones in marine fish larvae is still inadequate. Greater knowledge will serve as the basis for developing microdiets that are easily accepted and digested by larvae from the onset of exogenous feeding. For this reason, the effects of different molecular forms of dietary nitrogen sources used in microdiets on tryptic activity in marine larvae should be investigated.

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